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(54) Title: NOVEL IMMUNOSUPPRESSANT PEPTIDES

(57) Abstract

Peptide compositions are provided which bind MHC molecules of interest and inhibit T cell activation. The peptides are of 4-25 amino acid residues in length, and have a core binding region comprising, in the direction from the N- to the C- terminus, a hydrophobic L-amino acid or amino acid mimetic, a spacer sequence of 2-6 residues and a Thr or Thr mimetic. At least one residue is a D-amino acid or an amino acid mimetic. The compositions may be used to treat diseases associated with particular Dr alleles, including autoimmune diseases such as rheumatoid arthritis.

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NOVEL IMMUNOSUPPRESSANT PEPTIDES

The present invention relates to the use of novel peptides which bind to major histocompatibility complex (MHC) molecules and modulate an immune response.

An integral part of the mammalian immune response is the ability of T cells to recognize protein antigens. The T cells are presented with processed antigen complexed with the MHC molecules expressed on the surface of certain cells. Antigen presentation appears to be a major, if not the primary, function of MHC molecules.

Both helper and cytotoxic T cells are believed to recognize antigens by interacting with MHC-antigen complexes. MHC Class I molecules (e.g., HLA-A, -B and -C molecules in the human system) are involved in generating a T-killer cell response, in which cells bearing the eliciting antigen are attacked. Class II molecules (HLA-DP, -DQ and -DR in humans) present antigen to T-helper cells, and thus control the expansion and maturation of a selected population of B cells, resulting in the production of antigen-specific antibodies. Thus, Class I and Class II MHC molecules play a critical role in regulating an individual's immune response to a protein antigen.

Autoimmune diseases are characterized by an immune response against "self" antigens. In essence, autoimmune disease results when an individual's immune response is no longer able to distinguish self antigens from non-self (foreign) antigens. It is thought that self-reactive B cells exist in the body for many potential autoantigens, but that the reactivity of the self-reactive B cells and other immune cells is controlled by MHC molecules.

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Several autoimmune diseases have been shown to be associated with
particular MHC alleles. One of the most notable associations has been a
rheumatoid syndrome involving the spinal column, ankylosing
spondylitis, and the allele HLA-B27. Another, the haplotype HLA-DR2. is
common in the autoimmune disease multiple sclerosis. Hashimoto's
disease, which affects the thyroid, tends to be associated with
HLA-DR5. Other studies have shown that individuals having HLA-DR1,
and/or DR4w4 and/or DR4w14 alleles are genetically predisposed towards
rheumatoid arthritis. Research suggests that the corresponding MHC
proteins play a key role in such diseases, perhaps by binding with a
self-antigen and presenting it to T cells.

Current treatment for autoimmune disease such as rheumatoid
arthritis consists primarily of treating the symptoms, but not
intervening in the etiology of the disease. Broad spectrum
chemotherapeutic agents are typically employed, which agents are often
associated with numerous undesirable side effects.

The inadequate treatments presently available for rheumatoid
arthritis illustrate the urgent need for agents to treat autoimmune
diseases that avoid nonspecific suppression of an individual's overall
immune response yet do not cause serious side effects. Such agents
should also be economical to produce and possess favourable
pharmacologic properties for example a relatively long half-life,
thereby facilitating lower dosages and/or less frequent admin-
istration. Peptides which inhibit HLA-DR restricted T cell activation
by binding to HLA-DR molecules, such as DR1, DR4w4, or DR4w14 offer
these advantages.

For example, as HLA-DR alleles have been associated with
rheumatoid arthritis, antigens which bind to the DR molecule may serve
as a source of binding peptides. Influenza hemagglutinin ("HA") is a
protein identified as specifially reacting with HLA-DR, and portions
thereof may be screened for reactivity to provide a sequence which
binds the appropriate DR molecule, such as DR1, DR4w4 or DR4w14, and
then screened for the ability to inhibit or otherwise modulate an
antigen-specific immune response such as might be associated with the
disease. The MHC molecule with which the peptide is being tested may be

isolated from cells which express it, or the cells may be used intact.
A large number of cells with defined MHC molecules, particularly MHC
Class II molecules, are known and readily available. A variety of assay
procedures may be employed in the screening protocol and are also well
known in the art. Typically, for instance, the peptide may be labelled,
e.g., with a radionuclide, enzyme, fluorescer, etc. and specific
binding activity determined.

Using the procedure outlined herein, a partial sequence of HA,
amino acid residues 307 to 319 (designated here as peptide A or
HA307-319), has been identified which binds to both DR1 and DR4w4 MHC
molecules. The sequence of this peptide is as shown in Sequence
Identifier No.1.

The biological activity of the peptide, i.e. the ability to
inhibit antigen-specific T cell activation, may also be assayed in a
variety of systems. In one cellular assay, an excess of peptide is
incubated with an antigen-presenting cell of known MHC expression,
(e.g., DR1) and a T cell clone of known antigen specificity (e.g.,
tetanus toxin 830-843) and MHC restriction (again, DR1), and the
antigenic peptide itself (i.e., tetanus toxin 830-843). The assay
culture is incubated for a sufficient time for T cell proliferation,
such as four days, and proliferation is then measured using standard
procedures, such as pulsing with tritiated thymidine during the last 18
hours of incubation. The percent inhibition, compared to the controls
which received no inhibitor, is then calculated.

The capacity of peptides to inhibit antigen presentation in an in
vitro assay may be correlated to the capacity of the peptide to inhibit
an immune response in vivo. In vivo activity may be determined in
animal models, for example, by administering an antigen known to be
restricted to the particular MHC molecule recognized by the peptide,
and the immunomodulatory peptide. T lymphocytes are subsequently
removed from the animal and cultured with a dose range of antigen.
Inhibition of stimulation is measured by conventional means, e.g.,
pulsing with [³H]-thymidine, and comparing to appropriate controls.

Once a peptide which binds to the selected MHC molecule is

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identified, a "core binding region" of the antigen may be determined by
sythesizing overlapping peptides or by employing N-terminal or
C-terminal deletions (truncations) or additions. By "core binding
region" as used herein is meant a sequence of amino acids or amino acid
mimetics important in maintaining a binding affinity of the peptide for
an MHC molecule. The core binding region may contain contact residues
having characteristics essential for binding. Thus, the core binding
region comprises those residues of the peptide that define the
specificity of the peptide-MHC interaction. In the determination of a
core binding region, a series of peptides with single amino acid
substitutions may be employed to determine the effect of electrostatic
charge, hydrophobicity, etc. on binding. For instance, with peptide A,
a series of positively charged (e.g., Lys or Arg) or negatively charged
(e.g., Glu) amino acid substitutions may be made along the length of
the peptide revealing different patterns of sensitivity towards various
DR molecules. For example, the DR4w4 molecule preferentially binds to
peptides that bear little or no electrostatic charge in a core region
consisting of residues 309-314 inclusive. Notably, substitution of Tyr
309 of peptide A with either Glu or Arg results in substantially
decreased binding affinity.

Within the core region, "critical contact sites", i.e., those
residues (or their functional equivalents) which must be present in the
peptide so as to retain the ability to bind an MHC molecule and inhibit
the presentation to the T cell, may be identified by single amino acid
substitutions, deletions, or insertions. In addition, one may also
carry out a systematic scan with a specific amino acid (e.g., Ala) to
probe the contributions made by the side chains of critical contact
residues. For example, Ala substitutions along the length of HA peptide
A reveals that binding to the DR1 molecule is relatively unaffected by
such substitutions, except for an Ala substitution of position Tyr-309.
Binding to DR4w4 is relatively unaffected by Ala substitutions, except
at positions Tyr-309 and Thr-314.

Peptides of the invention which are relatively insensitive to
single amino acid substitutions with neutral amino acids, except at
essential contact sites, have been found to tolerate multiple
substitutions. Particularly preferred multiple substitutions are small,

relatively neutral moieties such as Ala, Gly, Pro, or similar residues.
The substitutions may be homo-oligomers or hetero-oligomers. The number
and types of residues which are substituted or added depend on the
spacing necessary between essential contact points and certain
functional attributes which are sought (e.g., hydrophobicity versus
hydrophilicity). Increased binding affinity for an MHC molecule may
also be achieved by such substitutions, compared to the affinity of the
parent peptide. In any event, such "spacer" substitutions should employ
amino acid residues or other molecular fragments chosed to avoid, for
example, steric and charge interference which might disrupt binding.

The effect of single amino acid substitutions may also be probed
using D-amino acids. Such substitutions may be made using well known
peptide synthesis procedures. Single D-amino acid substitutions in
peptide A reveal that those made to the core region produce a peptide
having substantially decreased affinity for MHC DR1, whereas those
D-form substitutions made outside the core region are relatively well
tolerated.

Using the strategies outlined above, certain minimal requirements
for peptide binding to HLA-DR have been identified. In particular, a
peptide of the present invention will comprise a core binding region
comprising a hydrophobic L-amino acid or amino acid mimetic, a spacer
sequence of from 2 to 6 residues, and a Thr or Thr mimetic residue,
wherein at least one residue is a D-amino acid or an amino acid
mimetic.

Accordingly, the present invention provides peptides of formula I
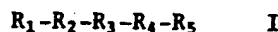
$$R_1-R_2-R_3-R_4-R_5 \qquad I$$

in which, reading from left to right in the direction from the N to the
C terminus;

  R_1 is a spacer sequence of from 0 to 7 amino acid or amino
acid mimetic residues,

  R_2 is a hydrophobic L-amino or L-amino acid mimetic residue,

  R_3 is a spacer sequence of from 2 to 6 L-amino acid or
L-amino acid mimetic residues,

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R_4 is Thr or a Thr mimetic residue, and

R_5 is a spacer sequence of between 0 and 10 amino acid or
amino acid mimetic residues,

provided that at least one residue is a D-amino acid or an amino acid
mimetic.

In certain embodiments, the peptides of the invention display at
least one of the following features 1) and 2):

1) if R_1 is present, the last residue of R_1, together with
R_2, constitutes an isostere of X_1-Phe;

2) R_3 comprises 2-5 residues, two of which constitute an
isostere of X_2-Gln;

wherein X_1 is a L- or D-amino acid mimetic and X_2 can be any
L-amino acid or L-amino acid mimetic.

In one preferred embodiment of the peptides according to the
invention, the last residue of R_1 (X_1) is positively charged. Preferred
residues are Ala and ala; the isostere of X_1-Phe is typically ala-Phe,
Ala-Phe, ala-Cha, Ala-Cha, ala-Ada or Ala-Ada, where Cha is cyclohexyl-
alanine and Ada is adamantylamine.

If an isostere of X_2-Gln is present, it is preferably Arg-Gln.

The term "spacer sequence" refers to sequences of amino acids or
amino acid mimetics, which are substantially uncharged or positively
charged under physiological conditions and which may have linear or
branched side chains.

By "amino acid mimetic" as used herein is meant a moiety, other
than a naturally occurring amino acid, that conformationally and
functionally serves as a substitute for a particular amino acid in a
peptide of the present invention without adversely interfering to a
significant extent with, or in some circumstances actually enhancing,
the interaction of the peptide with its MHC receptor. Peptides
incorporating amino acid mimetics may be synthesized and screened for

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effect on binding in a variety of assays, e.g., in a competition assay
with an unsubstituted parent peptide. However, the term "Thr mimetic",
or in general "X mimetic" where X is any specific natural amino acid,
includes other natural amino acids which may substitute for the
specific amino acid without adversely affecting binding, as described
above. For a general discussion of peptide mimetics, see, Morgan and
Gainor, Ann. Repts. Med. Chem. 24:243-252 (1989).

The term "residue" refers to an amino acid or amino acid mimetic
incorporated in a polypeptide by an amide bond or amide bond mimetic.

The term "isostere" as used herein refers to a sequence of two or
more residues that can be substituted for a second sequence because the
steric conformation of the first sequence fits a binding site specific
for the second sequence. The term specifically includes peptide back-
bone modifications (i.e., amide bond mimetics) well known to those
skilled in the art. Such modifications include modifications of the
amide nitrogen, the α-carbon, amide carbonyl, complete replacement of
the amide bond, extensions, deletions or backbone crosslinks. Several
peptide backbone modifications are known, including $\psi[CH_2S]$, $\psi[CH_2NH]$,
$\psi[CSNH_2]$, $\psi[NHCO]$, $\psi[COCH_2]$ and $\psi[(E)$ or (Z) $CH=CH]$. In the nomen-
clature used above, ψ indicates the absence of an amide bond. The
structure that replaces the amide group is specified within the
brackets.

Other possible modifications include an N-alkyl (or aryl)
substitution ($\psi[CONR]$), backbone crosslinking to construct lactams and
other cyclic structures, or retro-inverso amino acid incorporation
($\psi[NHCO]$). By "inverso" is meant replacing L-amino acids of a sequence
with D-amino acids, and by "retro-inverso" or "enantio-retro" is meant
reversing the sequence of the amino acids ("retro") and replacing the
L-amino acids with D-amino acids. For example, if the parent peptide is
Thr-Ala-Tyr, the retro modified form is Tyr-Ala-Thr, the inverso form
is thr-ala-tyr, and the retro-inverso form is tyr-ala-thr. Compared to
the parent peptide, a retro-inverso peptide has a reversed backbone
while retaining substantially the original spatial conformation of the
side chains, resulting in a retro-inverso isomer with a topology that
closely resembles the parent peptide and is able to bind the selected

MHC molecule. See Goodman et al. "Perspectives in Peptide Chemistry"
pp. 283-294 (1981).

    Various amino acid mimetics (as defined above) may be incorporated
in the isostere. The side chain of the individual residues may also be
modified in a number of ways. For instance, the side chain of a lysine
residue isostere may be lengthened or shortened. Other modifications
include the alkylation of NH_2 of lysine with a dihydroxypropyl radical.
The isosteres described above may comprise either a peptide bond or a
peptide bond mimetic, but preferably comprise a peptide bond.

    It should be understood that the nomenclature used to describe
peptide compounds follows the conventional practice wherein the amino
group is presented to the left (the N-terminus) and the carboxy group
to the right (the C-terminus) of each amino acid residue. In relation
to a sequence or part of a sequence, the term "first" refers to the
residue nearest to the N-terminus, and the term "last" to that nearest
the C-terminus. In the formulae representing specific embodiments of
the present invention, the amino- and carboxy-terminal groups, although
not specifically shown, will be understood to be in the form they would
assume at physiologic pH values, unless otherwise specified.

    In the formulae, each amino acid residue is generally represented
by the standard designation, in which the L-form of an amino acid
residue is represented by a three-letter symbol having a capital first
letter and the D-form for those amino acids having D-forms is
represented by a three-letter symbol having a lower case initial
letter. Glycine has no asymmetric carbon atom and is simply referred to
as "Gly". It is to be understood that where a sequence is defined as
consisting only of L-amino acids, that sequence may contain Gly, or
other amino acids lacking an asymmetric carbon atom.

    The peptides of the invention contain at least one residue
selected from D-amino acids and amino acid mimetics, preferably at
least one D-amino acid. Such residues are preferably located at one or
both of the termini, as this is found to improve the stability of the
peptide in vivo. The peptides of the present invention preferably have
a human serum half life at least about two times that of peptide A,

preferably about four to ten times greater. Peptides with half lives
greater than ten times that of peptide A are particularly preferred.
Stability can be assayed in a number of ways. For instance, peptidases
and various biological media, such as human plasma and serum, have been
used to test stability. A preferred method for determining stability is
to measure half life in the 25% human serum assay described in detail
below.

Certain preferred embodiments of the peptides of the present
invention comprise modifications to the N- and C-terminal residues to
alter the physical or chemical properties of the peptide, such as, for
example, to affect binding, stability, bioavailability, ease of
linking, etc.

The N-terminal residue can be modified to include alkyl,
cycloalkyl, aryl, arylalkyl, and acyl groups. The N-terminal residue
may be linked to a variety of moieties other than amino acids such as
polyethylene glycols (such as tetraethylene glycol carboxylic acid
monomethyl ether), pyroglutamic acid, succinoyl, methoxy succinoyl,
benzoyl, phenylacetyl, 2-, 3-, or 4-pyridylalkanoyl, aroyl, alkanoyl
(including acetyl and cycloalkanoyl e.g. cyclohexylpropanoyl),
arylakanoyl, arylaminocarbonyl, alkylaminocarbonyl, cycloalkyl-
aminocarbonyl, alkyloxycarbonyl (carbamate caps), and cycloalkoxy-
carbonyl, among others.

Preferred modifications of the C-terminus include modification of
the carbonyl carbon of the C-terminal residue to form a carboxy-
terminal amide or alcohol (i.e., as reduced form). In general, the
amide nitrogen, covalently bound to the carbonyl carbon on the
C-terminal residue, will have two substitution groups, each of which
can be hydrogen, alkyl or an alkylaryl group (substituted or
unsubstituted). Preferably the C-terminal is an amido group, such as
-$CONH_2$, -$CONHCH_3$, -$CONHCH_2C_6H_5$ or -$CON(CH_3)_2$, but may also be 2-, 3-,
or 4-pyridylmethyl, 2-, 3-, or 4-pyridylethyl, carboxylic acid, ethers,
carbonyl esters, alkyl, arylalkyl, aryl, cyclohexylamide, piperidine-
amide and other mono or distubstituted amides. Other moieties that can
be linked to the C-terminal residue include piperidine-4-carboxylic
acid or amide and cis- or trans- 4-amino- cyclohexanecarboxylic acid or

amide.

In forming C-terminal amidated compounds of the present invention, the compound can be synthesized directly, for example, using an amine and an oxime resin, as described by Kaiser et al., Science 243:187 (1989), or using a methyl benzhydryl amine (MBHA) polystyrene resin. Alternatively, the amidated compounds may be chemically amidated subsequent to peptide synthesis using means well known in the art, or enzymatically amidated. C-terminal alcohols and corresponding ethers can be prepared by methods well known to those skilled in the art. Fully protected peptides suitable for C-terminal amidation are conveniently prepared by solid phase synthesis using the SASRIN resin in conjunction with an FMOC synthesis protocol.

A typical synthesis procedure is as follows. The carboxy-terminal acid is coupled to a Sasrin resin (Bachem, Switzerland) using dicyclohexylcarbodiimide and 4-dimethylaminopyridine. Subsequent chain elongation is carried out using an Applied Biosystems 430A peptide synthesizer. After removal of the fluorenylmethyloxycarbonyl (Fmoc) protecting group from the alpha-amino group of the resin-bound amino acid by treatment with a 50 % solution of piperidine in dimethyl-formamide, the resin is coupled with a four fold excess of the hydroxybenzotriazole ester of the appropriate amino acid. Chain elongation is continued according to the same protocol. The final amino acid is incorporated with an alpha-amino-tert-butoxycarbonyl (t-BOC) protecting group. The peptide is then cleaved from the Sasrin resin by treatment with 1 % trifluoroacetic acid in dichloromethane. Upon neutralization with pyridine and evaporation of the solvent, a fully protected peptide with a free carboxy terminus is obtained. This compound is then coupled to an amine of choice using hydroxy-benzotriazole and dicyclohexylcarbodiimide as the condensing agents. Finally, amino terminal and side chain protecting groups are removed by treatment with trifluoroacetic acid.

In a preferred embodiment, the group R_1 contains 1 or 2 residues, with the first residue being a D-amino acid, preferably Ac-ala, phe or ala, wherein the prefix Ac- before the three-letter symbol for an amino acid signifies an acetylated N-terminus on that amino acid. The second

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residue, if present, is preferably Ala or ala. The group may also comprise at least one N-alkyl or α-alkyl amino acid residue. Particularly preferred groups R_1 are ala, ala-Ala, Ac-ala and Ac-ala-Ala.

R_2 is a hydrophobic residue and is thought to be a binding site contact residue. Although its selection is generally dependent on the particular MHC molecule of interest, the R_2 residue is preferably selected from the group of Tyr, Phe, Trp, Met, and their amino acid mimetics, such as β-2-thienyl-alanine, α-amino-4-phenylbutyric acid, phenylglycine, 3-(2-naphthyl)alanine, 3-(1-naphthyl)alanine, β-1-adamantylalanine, β-9-anthracenylalanine, 3-cyclohexylalanine, 2-amino-3(1,1'-biphenyl-4-yl)-propanoic acid, 2-aminoindane-2-carboxylic acid, tetrahydroisoquinoline carboxylic acid, 1-(2-propanoic acid)-3-amino-benzazepine-2-one, and 3-(4-pyridyl)-alanine. More preferably, particularly when the MHC molecule is DR1, DR4w4, and DR4w14, R_2 is Phe, cyclohexylalanine, or β-1-adamantylalanine. If R_1 is absent, R_2 is preferably cyclohexylpropanoyl.

R_3 usually comprises 4 residues, preferably composed of Gln-Ser-Gln-Thr, Gln-Arg-Gln-Thr, Ala-Arg-Ala-Thr, Ala-Ala-Ala-Thr, Ala-Ala-Ala-Ala, Ala-Ala-Ala-Arg, Ala-Ala-Ala-Lys or Ala-Asp-Ala-Ala, of which the last four are particularly preferred.

R_4 is also thought to be a binding site contact residue. It is preferably Thr, but may also be a Thr mimetic, such as His, Gly, Val, Pro, Ser, alkyl substituted His, hydroxyproline, hydroxyvaline, 3-acetyl-2,3-diaminopropanoic acid, 4-acetyl-2,4-diaminobutanoic acid, allothreonine, and the like.

R_5 is preferably 3-6 residues long, more preferably 4-5 residues long, the last residue being a D-amino acid or an unnatural amino acid not found in proteins. Preferred sequences are Lys-Ala-Ala-Pip-NH$_2$, Ala-Ala-Ala-Ala-ala, Lys-Ala-Ala-Ala-ala, Leu-Lys-Leu-Ala-thr, Leu-Lys-Ala-Ala-ala, and Ala-Lys-Ala-Ala-ala, wherein Pip-NH$_2$ is piperidine-4-carboxamide.

The peptide has a core binding region composed only of L-amino

acids. The core binding region will typically include, in the direction
from the N- to the C-terminus, a hydrophobic L-amino acid (preferably
Phe) or L-amino acid mimetic, a spacer sequence of from 2 to 6
residues, and a Thr or Thr mimetic residue, corresponding to R_2, R_3 and
R_4 respectively. Preferred sequences of the core binding region are
Cha-Ala-Ala-Ala-Ala-Thr, Cha-Ala-Ala-Ala-Lys-Thr,
Phe-Ala-Ala-Ala-Ala-Thr, Phe-Ala-Ala-Ala-Lys-Thr, or
Phe-Gln-Arg-Gln-Thr-Thr, where Cha is cyclohexylalanine.

R_1, R_3, and R_5, as previously described, are spacer sequences.
They comprise neutral and positively charged residues. The neutral
residues of the spacer sequences typically include, among others, Ala,
ala, Gly, Pro, β-Ala, substituted β-ala (e.g. α-methyl, β-methyl,
α-benzyl, or β-benzyl substituted), PABA, or other neutral spacers of
non-polar amino acids (those with hydrophobic side groups at
physiologic pH values) such as aminoisobutyric acid (Aib), Val, Leu,
Ile, Met, Phe, or Trp; or other neutral polar amino acids (those
residues with hydrophilic, uncharged side groups at physiologic pH),
such as Ser, Thr, Asn, Gln. A spacer sequence may also include amino
acid mimetics such as ethylene glycol or propylene glycol.

Positively charged residues may be incorporated in the spacer
sequences to increase aqueous solubility. The presence of these
residues generally increases solubility to at least about 1μg/ml and in
some cases, higher than 1mg/ml at pH 7.4. Positively charged spacer
residues include, among others, Lys, homo-Lys, Arg, N-alkyl or
N,N-dialkyl-Lys, homo-Arg, N-,N-dialkyl-homo-Arg, N-alkyl or
N,N-dialkyl-Arg, N,N,N-trialkylammonium salts, ornithine (Orn),
2,3-diaminopropanoic acid (dap), 2,4-diaminobutanoic acid (dab), and
may have, for example, molecules such as dimers of ethylene glycol
(polyethylene glycol or propylene glycol (polypropylene glycol)
attached thereto, e.g., to Lys, to further enhance solubility.

R_3 is a spacer sequence within the core binding region. As
discussed above, the core binding region (R_2 to R_4) of a peptide of the
present invention does not contain D-amino acids. Thus, the above
description of the residues in R_1 and R_5 applies to R_3 except that all
the R_3 amino acid residues are in the L form.

In addition to the significances given above for R_2, R_2 may, if R_1 is absent, be any of a number of non-amino acid hydrophobic moieties which form a hydrophobic "cap" at the N-terminus. These moieties are preamino acid mimetics which do not interfere with recognition of the peptide by the MHC molecule and increase serum half life by increasing peptidase resistance of the peptide.

The N-terminal hydrophobic moiety can be linked to the amino terminus (e.g., an R_1 residue; R_2, if R_1 is absent; or R_3, if the hydrophobic moiety is R_2) through any of a number of linkages such as peptide backbone modifications known to those skilled in the art (see, Spatola, Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, Vol. VII (Weinstein ed., 1983). Linkages suitable in the present invention include:

$$R-\ ,\ R-CO-\ ,\ R-CS-\ ,\ R-SO_2-\ ,\ R-O-CO-\ ,\ R-NH-CO-\ ,\ R-O-CS-\ ,$$

$$R-S-CO-\ ,\ R-NH-CS-\ ,\ \text{and}\ \ R-S-CS-\ ,$$

where R is the hydrophobic moiety.

A wide variety of N-terminal caps (e.g. amino acid mimetics) can be used so long as MHC binding is not affected and serum half life is improved. Examples of suitable N-terminal moieties include aryl (including phenyl, 1-naphthyl, and 2-naphthyl); arylC_{1-7}alkyl; styryl; C_{4-8}cycloalkyl; and C_{4-8}cycloalkylC_{1-7}alkyl groups, which may be mono-, di-, or tri-substituted by substituents selected from hydroxy, methoxy, ethoxy, phenoxy, amino, dimethylamino, chloro, bromo, methyl and methylthio; adamantyl and adamantylC_{1-7}alkyl moieties.

Further N-terminal caps include groups of formula II

$$R'-(CH_2)_n- \hspace{4cm} \text{II}$$

in which R' is a heterocyclic group selected from pyridyl, pyrazinyl, triazinyl, thiazolyl, oxazolyl, imidazolyl and isoxazolyl, which may be mono- or di-substituted by substituents selected from hydroxy, methoxy,

ethoxy, phenoxy, amino, dimethylamino, chloro, bromo, methyl and
methylthio; and n = 0 to 7.

Peptides of the present invention possess various combinations of
the above features. For instance, virtually any residue of peptide A
may be substituted with one of the D-amino acids or amino acid mimetics
discussed above, although, as discussed above, the core binding region
should consist exclusively of L-amino acids. Alternatively, one or both
terminal residues may be modified as described above. In certain
embodiments both terminal residues are modified D-amino acids, for
instance, the C-terminal residue is amidated and the N-terminal residue
is acetylated. In addition, the C-terminal residue can be an isostere
of the dipeptide Ala-Ala (such as piperidine-4-carboxylic acid or amide
and cis-4-aminocyclohexanecarboxylic acid or amide) and the N-terminal
residue can be a D-amino acid. In other embodiments R_1 may be absent
and R_2 is a hydrophobic amino acid mimetic (e.g. cyclohexylpropanoyl)
which forms a hydrophobic cap. Any combination of the modifications and
substitutions discussed can be used so long as MHC binding is not
adversely affected.

Peptides or analogs thereof having the desired activity may be
modified as necessary to provide certain desired attributes, e.g.,
improved pharmacological characteristics, while increasing or at least
retaining substantially all of the biological activity of the
unmodified peptide to inhibit the preselected immune response. For
instance, the peptides can be modified by extending, decreasing or
substituting in the compound's amino acid sequence, e.g., by the
addition or deletion of amino acids on either the amino terminal or
carboxy terminal end, or both, of the sequences disclosed above.

The peptides or analogs of the invention can be modified by
altering the order or composition of certain residues, it being readily
appreciated that certain amino acid residues essential for biological
activity, e.g., those at critical contact sites, may generally not be
altered without an adverse effect on biological activity. The
non-critical amino acids need not be limited to those naturally
occurring in proteins, such as L-α-amino acids, or their D-isomers, but
may include non-protein amino acids as well, such as β-, γ-, or δ-amino

acids, β-, γ-, or δ-imino acids (such as piperidine-4-carboxylic acid)
as well as many derivatives of L-α-amino acids. As discussed, a peptide
of the present invention may generally comprise either L-amino acids or
D-amino acids, but not D-amino acids within the core binding region.

The peptides and analogs thereof of the present invention which
bind to MHC molecules and inhibit or block MHC restricted antigen-
specific T cell activation comprise at least 4 amino acid residues or
the conformational equivalent thereof, more usually at least 6,
preferably at least 8 and more preferably at least 11 residues long.
They usually will not exceed 25 residues or the equivalent thereof in
length, preferably no more than 18, more preferably 14 amino acid
residues or the equivalent thereof in length. As mentioned above, it is
understood that non-critical amino acids may be substituted with other
amino acids or with amino acid mimetics; in instances where spacer
molecules are substituted for non-essential residues, the approximate
length of the resulting peptide should generally not exceed that which
may be accommodated by the binding domain of the selected MHC
molecule.

The peptides of the present invention and pharmaceutical
compositions thereof are particularly useful for administration to
mammals, particularly humans, to treat a variety of conditions
involving autoimmune diseases. For instance, although rheumatoid
arthritis can be treated with conventional chemotherapeutic agents as
mentioned above, the peptides of the invention as described herein may
be used to treat the symptoms of rheumatoid arthritis or prevent their
occurrence in identified patients or those patients at risk of
developing the disease. Since the immunomodulatory peptides which bind
to selected MHC molecules are more selective than conventional agents,
and generally will be less toxic, they will be more effective and less
likely to cause complications than the conventional broad spectrum
agents. Of course, if necessary or desirable, the peptides may be
administered in conjunction with the traditional chemotherapeutic
agetns, and may be used to lower the dose of such agents to levels
substantially below those typically associated with toxicity.

Other autoimmune associated disorders for which the peptides of

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the invention may be employed to relieve the symptoms of, treat or
prevent the occurrence or reoccurrence of include, for example, Sjogren
syndrome, scleroderma, polymyositis, dermatomyositis, systemic lupus
erythematosus, juvenile rheumatoid arthritis, ankylosing spondylitis,
myasthenia gravis (antibodies to acetylcholine receptors), bullous
pemphigoid (antibodies to basement membrane at dermalepidermal
junction), pemphigus (antibodies to mucopolysaccharide protein complex
or intracellular cement substance), glomerulonephritis (antibodies to
glomerular basement membrane), Goodpasture's syndrome, autoimmune
hemolytic anemia (antibodies to erythrocytes), Hashimoto's disease
(antibodies to thyroid), pernicious anemia (antibodies to intrinsic
factor), idiopathic thrombocytopenic purpura (antibodies to platelets),
Grave's disease and Addison's disease (antibodies to thyroglobulin),
and the like.

The dose of the immunomodulatory peptides of the invention for
treatment of autoimmune disease will vary according to, e.g., the
peptide composition, the manner of administration, the particular
disease being treated and its severity, the overall health and
condition of the patient, and the judgment of the prescribing
physician. For example, for the treatment of rheumatoid arthritis with
a peptide of the present invention the dose is in the range of about 50
μg to 2,000 mg/day, preferably 5 to 700 mg/day for a 70 kg patient.
Administration should begin at the first sign of symptoms or shortly
after diagnosis, and continue at least until symptoms are substantially
abated and for a period thereafter. In established cases loading doses
followed by maintenance doses may be required.

The pharmaceutical compositions are intended for parenteral,
topical, oral or local administration, such as by aerosol or trans-
dermally, for prophylactic and/or therapeutic treatment. The compos-
itions are suitable for use in a variety of drug delivery systems.
Preferably, the pharmaceutical compositions are administered parent-
erally, e.g. intravenously. Thus, the invention provides compositions
for parenteral administration which comprise an immunomodulatory
peptide dissolved or suspended in an acceptable carrier, preferably an
aqueous carrier e.g. water, buffered water, 0.4% saline, 0.3% glycine,
hyaluronic acid and the like. These compositions may be sterilized by

conventional sterilization techniques, or may be sterile filtered. The
resulting aqueous solutions may be packaged for use as is, or
lyophilized, the lyophilized preparation being combined with a sterile
aqueous carrier prior to administration. The compositions may contain
pharmaceutically acceptable auxiliary substances as required to
approximate physiological conditions, such as pH adjusting and
buffering agents, tonicity adjusting agents, wetting agents, detergents
and the like, for example, sodium acetate, sodium lactate, sodium
chloride, potassium chloride, calcium chloride, sorbitan monolaurate,
triethanolamine oleate, etc.

The concentration of immunomodulatory peptides in the
pharmaceutical formulations can vary widely, i.e., from less than about
0.01%, usually at or at least about 5% to as much as 50 to 75% by
weight and will be selected primarily by fluid volumes, viscosities,
etc., in accordance with the particular mode of administration
selected. Thus, a typical pharmaceutical composition for intravenous
infusion could be made up to contain 250 ml of sterile Ringer's
solution, and 10 mg of peptide. Two or more peptides of the invention
may be combined to form a peptide "cocktail" under certain
circumstances for increased efficacy. The peptides of the invention may
also be used in conjunction with other pharmaceutically active agents.

For solid compositions, conventional nontoxic solid carriers may
be used which include, for example, pharmaceutical grades of mannitol,
lactose, starch, magnesium stearate, sodium saccharin, talcum,
cellulose, glucose, sucrose, magnesium carbonate, and the like. For
oral administration, a pharmaceutically acceptable nontoxic composition
is formed by incorporating any of the normally employed excipients,
such as those carriers previously listed, and generally 10-95% of
active ingredient, that is, one or more peptides of the invention,
preferably 25-75%.

For aerosol administration, the immunomodulatory peptides are
preferably supplied in finely divided form along with a conventional
non-toxic surfactant and a suitable propellant. Typical percentages of
peptides are 0.01%-20% by weight, preferably 1%-10%; and of surfactant
from 0.1%-20% by weight, preferably 0.25%-5%.

The compositions containing the immunomodulatory peptides can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a disease, as described above, in an amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on the severity of the disease and the weight and general state of the patient, but generally range from about 0.1 mg to about 2,000 mg of peptide per day for a 70 kg patient, with dosages of from about 0.5 mg to about 700 mg of peptide per day being more commonly used. It must be kept in mind that the materials of the present invention may generally be employed in serious disease states, that is, life-threatening or potentially life threatening situations.

In prophylactic applications, compositions containing the peptides of the invention are administered to a patient susceptible to or otherwise at risk of a particular autoimmune disease to enhance the patient's own immunoregulatory capabilities. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight, but generally range from about 0.1 mg to about 500 mg per 70 kg patient, more commonly from about 0.5 mg to about 200 mg per 70 kg of body weight.

The peptides may also find use as diagnostic reagents. For example, a peptide may be used to determine the susceptibility of a particular individual to a treatment regimen which employs the peptide or related peptides, and thus may be helpful in modifying an existing treatment protocol or in determining a prognosis for an affected individual. In such instances cells bearing the selected MHC antigen, e.g., white blood cells, will be obtained from the individual, incubated in vivo with the peptide(s), an immunostimulatory peptide, and a collection of T cells from the same individual. The proliferation of the T cells can then be assayed in the presence or absence of said immunomodulatory peptide or peptide mixture.

EXAMPLES

Selection and Screening of Peptide Library

This Example illustrates the method used to identify peptides which bind human MHC HLA-DR1 and -DR4w4. A library of about one hundred eighty synthetic peptides is tested. The library screened is non-redundant in the sense that care is taken to eliminate amino acid sequences too closely related; overlapping peptides are allowed but only if they overlap less than 50% of total residues.

Cells. EBV-transformed homozygous cell lines are used as a source of DR molecules. Cell lines are routinely monitored for DR expression by FACS analysis. Their DR types are confirmed by serological typing (Terasaki et al., Amer. J. Clin. Path. 69:103-120 (1978)) and RFLP analysis (Schreuder, J. Expt. Med. 164:938 (1986)). Cell lines used are maintained in vitro by culture in RPMI 1640 medium (Flow Labs, McLeon, VA), supplemented with 2 mM L-glutamine (Gibco, Grand Island, NY), 50 mM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO), 100 µg/ml streptomycin, 100 U/ml penicillin (Gibco, Grand Island, NY), and 10% heat-inactivated fetal calf serum (Hazelton Biologics Inc., Lenexa, KS) or horse serum (Hazelton Biologics Inc.). Large quantities of cells are grown in stationary cultures (250 cc flasks). Cells are lysed at a concentration of 10^8 cells/ml in 50 mM Tris-HCl pH 8.5, containing 2% Rennex, 150 mM NaCl, 5 mM EDTA, and 2 mM PMSF. The lysates are cleared of nuclei and debris by centrifugation at 10,000 x g for 20 minutes.

Affinity purification of DR molecules. DR molecules are purified essentially as described by Gorga et al. (Gorga et al., J. Biol. Chem. 262:16087-16094 (1987)) using the monoclonal antibody LB3.1 (Gorga et al., Cell. Immunol. 103:160-173 (1986)), covalently coupled to protein A-Sepharose CL-4B. Epstein-Barr virus-transformed human B cell lines, homozygous at the DR locus, are used as a source of DR molecules. The LG-2 (DR1) and Preiss (DR4, Dw4) lines were obtained from Dr. J.C. Gorga (Harvard University, Cambridge, MA), and MAT (DR3), Beh (DR4, Dw4) and SWEIG (DR5) from Dr. G. Nepom (Virginia Mason Research Center, Seattle, WA). Aliquots of cell lysates equivalent to approximately 10 g

of cells are passed sequentially through the following columns:
Sepharose CL-4B (10 ml), protein-A-Sepharose (5 ml), W6/32-protein-A-
Sepharose (10 ml), LB3.1-protein-A-Sepharose (15 ml), using a flow rate
of 30 ml/h. The columns are washed with 10 column volumes of 10 mM
Tris-HCl pH 8.0, 0.1% Rennex (5 ml/hr; 2 column volumes of PBS and 1%
octylglucoside. The DR is eluted with 0.05 M diethylamine in 0.15 M
NaCl containing 1% octylglucoside (pH 11.5), immediately neutralized
with 2 M glycine pH 2.0 and concentrated by ultrafiltration through an
Amicon YM-30 membrane. Protein content is evaluated by a BCA protein
assay (Pierce) and confirmed by SDS PAGE electrophoresis.

Peptide synthesis. Peptides are synthesized on an Applied
Biosystems (Foster City, CA) 430A peptide synthesizer according to well
known protocols. The synthesis of the peptide
ala-Cha-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Ala-alaNH$_2$ (Example 10 below)
is typical. The hydroxybenzotriazole ester of tert-butyloxycarbonyl-d-
alanine (Boc-d-alanine) is prepared by treatment of Boc-d-alanine with
equimolar amounts of hydroxybenzotriazole and dicyclohexylcarbodiimide
in dimethylformamide for thirty minutes at room temperature. The
mixture is then filtered to remove precipitated dicyclohexylurea. The
resulting solution is added to a methylbenzhydrylamine resin and
allowed to react for 60 minutes with gentle shaking or rocking. The
resin is thoroughly washed with dimethylformamide followed by
dichloromethane. The t-butyloxycarbonyl protecting group of the
resin-bound alanine is then removed by mixing the resin together with a
50 % solution of trifluoroacetic acid in dichloromethane for 30 min.,
followed by thorough washing with dichloromethane. The resulting resin-
bound amine trifluoroacetate salt is neutralized by treatment with a 20
% solution of diisopropylethylamine in dichloromethane.

Chain elongation is accomplished by repeating the above sequence
of 1) amino acid activation, 2) coupling and 3) deprotection for each
amino acid residue. Upon completion of the chain elongation, the resin
is dried and the peptide is cleaved from the resin and completely
deprotected by exposure to hydrogen fluoride in the presence of anisole
for 90 minutes at 0°C. The hydrogen fluoride is evaporated with a
nitrogen stream and the peptide is washed with cold diethylether.
Finally the peptide is redissolved in 25 % aqueous acetic acid and the

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solvent is removed by lyophilization. The synthesis of peptides containing amino acid mimetics is carried out in the same way. Suitably protected amino acid mimetics are incorporated according to the above procedures. The peptides are then purified by reverse-phase high-performance liquid chromatography (HPLC). The purity of the peptides is substantiated by amino acid sequence and/or composition analysis. They are routinely >95% pure after HPLC. Peptides are radiolabeled as described below.

DR-peptide binding assay. Purified DR molecules (10-1,000 nM) are incubated with 10 nM ^{125}I-radiolabeled peptide for 48 hours in the presence of added protease inhibitors. HPLC-purified peptides are radioiodinated (^{125}I) using the Chloramine T method, (Buus et al., Science 235:1353-1358 (1987)). The final concentrations of protease inhibitors are: 1 mM PMSF, 1.3 mM 1,10-phenanthroline, 73 µM pepstatin A, 8 mM EDTA, 6mM N-ethyl maleimide, and 200 µM Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK). The final detergent concentration in the incubation mixture is 0.05% NP-40. Several of the peptides screened are hydrophobic and require DMSO to maintain solubility and reduce peptide adsorption to surfaces. In these instances peptide stock solutions are prepared in neat DMSO, and the final DMSO concentration in the incubation mixture is adjusted to 5%. Control experiments demonstrate that the ID 50% values obtained with or without DMSO are similar. The DR-peptide complexes are separated from free peptide by gel filtration on a Sephadex G50 (Pharmacia Fine Chemicals, Piscataway, NJ) column 23 x 1.3 cm), as previously described. Id. The columns are eluted using PBS (0.5% NP-40, 0.1% NaN$_3$) collected in 1 ml fractions, which are then assayed for radioactivity. The fraction of peptide bound to DR (α) relative to the total amount of offered peptide is determined as the ratio of peptide in the void volume to the total peptide recovered. The α is corrected by subtracting the negative control (^{125}I-peptide incubated for two days at room temperature in the presence of protease inhibitors and 120 µg/ml of unlabeled peptide, but in the absence of any DR). Competitive inhibition by unlabeled peptide is used to determine ID 50% values.

Peptide HA 307-319, ("Peptide A") having good binding activity for both DR1 and DR4w4 molecules, is chosen from the screening assays for

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further study. The DR1, DR2, Dr4v4 and DR4v14 binding capacities of a
series of peptide A analogs with single or multiple amino acid
substitutions are determined as described above. In addition, the
peptides are tested for their ability to block an antigen-specific,
HLA-DR restricted T cell proliferative response. The protocol is as
follows:

Cellular Assay for Inhibition of Antigen Presentation

An EBV positive, DR1+ homozygous typing cell line, LK-2, is washed
3X with Hank's Balanced Salt Solution (HBSS), suspended to 5×10^6/ml
in HBSS, and fixed with 0.5% p-formaldehyde (v/v) at room temperature
for 20 minutes. The fixed LG-2 cells are washed 1X with HBSS, washed 2X
with RPMI media supplemented with L-glutamine, non-essential amino
acids, sodium pyruvate, antibiotics, and 10% human sera type AB
(Complete Media, CM), resuspended at 10^6/ml in CM, and then plated as
100 µl into individual wells of a 96 well microtiter dish. The fixed
LG-2 cells are then pulsed simultaneously for 2 hours at 37°C with 50
µl of test peptide and 50 µl of stimulatory peptide which have been
dissolved in CM. The amount of stimulatory peptide added to each test
well is constant and is calculated so as to yield 60-80% of the maximal
T cell proliferative response. The potential inhibitor peptide is
evaluated in duplicate at several concentrations corresponding to
multiples of the stimulatory peptide concentration, usually 3X, 10X,
50X, and 250X. At the end of the two hour incubation the plates are
centrifuged, and the media carefully aspirated and replaced with fresh
medium. The plates are washed in this manner three times in order to
ensure complete removal of unbound peptide. At the end of the washes 50
µl of CM remains in the well. In separate wells, increasing concen-
trations of stimulatory peptide are assayed in the absence of
inhibitory peptide in order to obtain dose-response curves for the
stimulatory peptide and to ensure that the amount of stimulatory
peptide used in test wells is truly limiting.

Antigen-specific T cells are washed 2X in CM, suspended to $2 \times$
10^5/ml in CM, and plated as 150 µl into the wells containing the
peptide-pulsed LG-2 cells. The cultures are incubated for three days at
37°C, and pulsed with 0.1 µCi/well of ^{3}H-thymidine during the last 16

hours of culture. At the termination of the cultures the cells are harvested onto glass fiber and the amount of ^3H-thymidine, which has been incorporated by the responder T cell, determined using liquid scintillation counting.

The inhibitory activity of each peptide is determined relative to the peptide of Example 29 below. For each test peptide the amount of inhibition is plotted as a function of the inhibitor concentration, and this graph is used to determine a 50% inhibitory dose. The inhibitory activity is then expressed as the ratio of the 50% inhibitory dose of the standard peptide (determined in a parallel experiment) to that of the test peptide. These data are presented in the last th ə columns of Table 1. This inhibitory value constitutes an additional means by which the MHC binding peptides can be compared.

Stability Assays

To analyze stability of the peptides, the half life of the peptides is determined using a 25% human serum (v/v) assay. Using the procedure described below, the 25% human serum half life of peptide A is determined to be about 100 minutes. The protocol is as follows. Pooled human serum (Type AB, non-heat inactivated) is obtained from Irvine Scientific and is delipidated by centrifugation before use. Human serum is diluted to 25% with RPMI tissue culture media. All chemicals (reagent or HPLC grade) are purchased commercially from Sigma or Aldrich and used without further purification.

Typically, 1 ml of reaction solution in a 1.5 ml Eppendorf tube is temperature-equilibrated at $37 \pm 1°C$ for 15 minutes before adding 5 μl of peptide stock solution (10 mg/ml in DMSO) to make the final peptide concentration 40μg/ml. The initial time is recorded. At known time intervals 100 μl of reaction solution is removed and added to 200 μl of either 6% aqueous trichloracetic acid or ethanol. The cloudy reaction sample is cooled (4°C) for 15 minutes and then spun at 14,000 rpm (Eppendorf centrifuge) for 2 minutes to pellet the precipitated serum proteins.

The following controls are carried out for each run: i) each set

of stability experiments includes a degradation study on a reference
study on a reference peptide (Peptide A), carried out in separate
solution, ii) peptide stability is also determined in precipitation
supernatant containing 4% (or greater) trichloroacetic acid to ensure
that the peptides do not undergo acid-catalyzed degradation while
awaiting HPLC sample analysis, iii) sample recovery upon TCA or ethanol
precipitation of serum proteins is determined by comparison of the
peptide peak area at time = 0 with a peptide stock solution of known
concentration in DMSO/H_2O (1:1). Generally, 6% TCA shows the highest
peptide recovery, but where less than 90% recovery in TCA is observed,
EtOH is used; all peptides show 85% or greater recovery in their
optimal precipitation media and iv) peptide degradation is carried out
under conditions where the degradation rate constant is independent of
peptide concentration (demonstrated, for example, by lowering the
peptide concentration or using radiolabeled peptide only), and
proportionately dependent on biological media concentration.

Peptide analysis is carried out by reversed-phase HPLC using
stability-specific chromatography conditions. Most samples are analyzed
using a 5 μm 25 x 0.4 cm Vydac C-18 column and a 0 —> 50 % gradient
(0-0.08 % trifluoroacetic acid in water —> 0.08 % trifluoroacetic acid
in CH_3CN) over 30 minutes (flow = 1 mL/minute, detection = 214 nm, AUFS
= 0.2) at room temperature. Where peptide coelutes with a media peak
or one of the peptide degradation product peaks, a different brand of
C-18 column (for example, Waters Bondapak) is used to effect
separation. HPLC linearity is determined for a representative group of
peptides up to 50 μg/mL (three-fold higher than the concentration
actualy assayed for in the biological media Examples) in DMSO/H_2O
(1:1). This mixed solvent system is chosen to minimize peptide loss
due to adhesion to the reaction vessel. HPLC stability-specificity is
carried out by traditional methods including: separate analysis of
non-precipitated media peaks, analysis of peptide peak shape and peak
absorbance ratioing at 50 % peptide remaining, and kinetic analysis
where first order loss of parent peptide should be observed
(degradation of the parent peptide cannot display a first order decay
curve [when run under pseudo first order conditions] if B or C [of same
retention time and similar molar absorptivity] build up to any
appreciable extent).

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Kinetic analysis is carried out by least squares analysis of log (integration peak area) versus time. When necessary, correction is made for small, interfering media peaks that coelute with the parent peptide (subtraction of background). Most reactions are followed for at least two halflives (except for the most stable peptides) and all pseudo first order plots are linear showing correlation coefficients > .98 (8 points).

EXAMPLES 1-48

The following peptides were synthesized and tested:

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Ex.No.	Sequence
1	ala-Ala-Cha-Ala-Ala-Ala-Ala-Thr-Leu-Lys-Ala-Ala-alaNH$_2$
2	tyr-Ala-Ala-Phe-Ala-Ala-Ala-Ala-Thr-Ala-Lys-Ala-Ala-alaNH$_2$
3	ala-Ala-Cha-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Ala-alaNH$_2$
4	tyr-Ala-Ala-Phe-Ala-Ala-Ala-Ala-Thr-Leu-Lys-Ala-Ala-alaNH$_2$
5	tyr-Ala-Ala-Cha-Ala-Ala-Ala-Ala-Thr-Leu-Lys-Ala-Ala-alaNH$_2$
6	tyr-Ala-Ala-Cha-Ala-Ala-Ala-Lys-Tyr-Ala-Ala-Ala-Ala-alaNH$_2$
7	Ac-tyr-Ala-Ala-Phe-Ala-Ala-Ala-Lys-Thr-Leu-Ala-Ala-Ala-alaNH$_2$
8	ala-Ala-Phe-Ala-Ala-Ala-Ala-Thr-Leu-Lys-Ala-Ala-alaNH$_2$
9	Ac-tyr-Ala-Ala-Cha-Ala-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Ala-alaNH$_2$
10	ala-Cha-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Ala-alaNH$_2$
11	ala-Ala-Phe-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Ala-alaNH$_2$
12	tyr-Ala-Ala-Phe-Ala-Ala-Ala-Lys-Tyr-Ala-Ala-Ala-Ala-ala
13	ala-Ala-Phe-Ala-Ala-Ala-Ala-Thr-Ala-Lys-Ala-Ala-alaNH$_2$
14	ala-Ala-Phe-Ala-Ala-Ala-Lys-Thr-Leu-Ala-Ala-Ala-alaNH$_2$
15	ala-Ala-Phe-Ala-Lys-Ala-Ala-Thr-Ala-Lys-Ala-Ala-alaNH$_2$
16	ala-Ala-Phe-Ala-Ala-Ala-Ala-Thr-Leu-Lys-Ala-Ala-ala
17	Ac-ala-Ala-Phe-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Phe-alaNH$_2$
18	Ac-ala-Ala-Cha-Ala-Ala-Ala-Ala-Thr-Leu-Lys-Ala-Ala-alaNH$_2$
19	Ac-ala-Ala-Cha-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Ala-alaNH$_2$
20	ala-Ala-Phe-Ala-Lys-Ala-Ala-Thr-Leu-Lys-Ala-Ala-alaNH$_2$
21	ala-Ala-Phe-Ala-Ala-Ala-Ala-Thr-Ala-Lys-Ala-Ala-ala
22	tyr-Ala-Ala-Cha-Ala-Ala-Ala-Ala-Thr-Lys-Ala-Ala-Ala-alaNH$_2$
23	Ac-tyr-Ala-Ala-Cha-Ala-Ala-Ala-Ala-Thr-Leu-Lys-Ala-Ala-alaNH$_2$
24	tyr-Ala-Ala-Cha-Ala-Ala-Ala-Ala-Hpr-Lys-Ala-Ala-Ala-alaNH$_2$
25	ala-Ala-Phe-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Phe-alaNH$_2$
26	tyr-Ala-Ala-Phe-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Phe-alaNH$_2$
27	ala-Ala-Cha-Ala-Ala-Ala-Ala-Hpr-Lys-Ala-Ala-Ala-alaNH$_2$
28	tyr-Ala-Ala-Phe-Ala-Ala-Ala-Lys-Thr-Leu-Ala-Ala-Ala-alaNH$_2$
29	ala-Ala-Phe-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Ala-alaNH$_2$
30	Ac-tyr-Ala-Ala-Phe-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Ala-ala
31	Ac-tyr-Ala-Ala-Phe-Ala-Ala-Ala-Ala-Thr-Ala-Lys-Ala-Ala-alaNH$_2$
32	Ac-tyr-Ala-Ala-Phe-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Phe-alaNH$_2$
33	Ac-tyr-Ala-Arg-Phe-Gln-Arg-Gln-Thr-Thr-Leu-Lys-Ala-Ala-ala
34	tyr-Ala-Ala-Phe-Gln-Arg-Gln-Thr-Thr-Leu-Lys-Ala-Ala-ala
35	Ac-tyr-Ala-Arg-Phe-Gln-Ser-Gln-Thr-Thr-Leu-Lys-Ala-Lys-thr
36	peg-Tyr-Ala-Arg-Phe-Gln-Ser-Gln-Thr-Thr-Leu-Lys-Ala-Lys-ThrNH$_2$
37	ala-Phe-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Ala-alaNH$_2$
38	tyr-Ala-Arg-Ada-Ala-Ser-Gln-Thr-Thr-Leu-Lys-Ala-Lys-thrNH$_2$
39	ala-Ala-Phe-Ala-Ala-Ala-Ala-Dpl-Lys-Ala-Ala-Ala-alaNH$_2$
40	Ada-Ala-Arg-Gln-Thr-Thr-Leu-Lys-Ala-Ala-alaNH$_2$
41	ala-Dpl-Phe-Ala-Ala-Ala-Ala-Hpr-Leu-Dpl-Ala-Ala-Tol
42	ala-Ala-Phe-Ala-Ala-Ala-Ala-Hpr-Ala-Dpl-Ala-Ala-Tol
43	tyr-Ala-arg-Ada-Ala-Arg-Gln-Thr-Thr-Leu-Lys-Ala-Ala-alaNH$_2$
44	ala-ala-Phe-Ala-Ala-Ala-Ala-Hpr-Leu-Dpl-Ala-Ala-Tol
45	ala-Dpl-Phe-Ala-Ala-Ala-Ala-Thr-Leu-Dpl-Ala-Ala-Tol
46	ala-Ala-Phe-Ala-Ala-Ala-Ala-Thr-Leu-Dpl-Ala-Ala-Tol
47	ala-Dpl-Phe-Ala-Ala-Ala-Ala-Thr-Ala-Dpl-Ala-Ala-Tol
48	ala-Ala-Cha-Ala-Ala-Ala-Ala-Pro-Lys-Ala-Ala-Ala-alaNH$_2$

Notes   Ac- at start signifies an acetylated N-terminus
             NH$_2$ at end signifies a carboxyamide modified C-terminus

Cha = L-cyclohexylalanine   Hpr = trans-4-hydroxyproline
Ada = L-adamantylalanine   Dpl = N-(dihydroxypropyl)-L-lysine
Tol = L-threoninol
peg = tetraethyleneglycol carboxylic acid monomethyl ether.

TABLE 1

Ex. No.	Stability t½ (min)[1]	MBC Binding Assay			Cellular Assay		
		DR1[2]	DR4w4[2]	DR4w14[3]	DR1[4]	DR4w[5]	DR4w14[4]
1	356	++	++	++	++	++	-+
2	240	++	++	++	++	++	++
3	265	++	++	++	++	++	-+
4	260	++	++	++	++	++	++
5	287	++	++	++	++	++	++
6	411	++	++	++	++	++	++
7	300	+	++	++	+	++	±
8	445	++	++	++	++	++	++
9	n.d.	++	++	++	++	++	+
10	383	++	++	++	++	++	+
11	750	++	++	++	++	++	++
12	531	++	++	++	++	++	+
13	335	++	++	++	+	+	++
14	495	++	++	++	+	++	+
15	575	++	++	++	++	++	+
16	246	++	++	++	+	++	+
17	627	++	++	++	+	++	+
18	314	++	++	++	++	++	n.d.
19	575	+	++	++	++	++	++
20	531	++	++	++	++	++	++
21	329	++	++	++	++	+	±
22	265	++	++	++	++	++	+
23	n.d.	++	++	++	++	+	n.d.
24	300	++	++	++	++	++	++
25	665	++	++	++	+	++	++
26	300	++	++	++	++	++	n.d.
27	216	++	++	++	++	++	+
28	226	++	++	++	+	++	+
29	687	++	++	++	+	+	+
30	406	±	++	++	+	++	±
31	n.d.	±	++	±	±	+	±
32	n.d.	+	++	++	+	+	±
33	400	+	++	++	+	+	±
34	252	++	++	++	+	+	+
35	265	++	++	+?	+	+	±
36	n.d.	±	++	++	+	+	±
37	560	+	++	++	±	++	n.d.
38	n.d.	++	++	++	++	++	n.d.
39	n.d.	±	++	±	++	++	n.d.
40	n.d.	++	++	++	±	++	n.d.
41	n.d.	+	++	++	+	++	n.d.
42	n.d.	++	++	++	+	++	n.d.
43	n.d.	++	++	++	±	++	n.d.
44	n.d.	++	++	++	+	++	n.d.
45	n.d.	++	++	++	++	++	n.d.
46	n.d.	++	++	++	+	++	n.d.
47	n.d.	±	++	++	+	+	n.d.
48	n.d.	++	++	±	++	±	n.d.

Notes to Table 1:

1) determined in 25% human serum

2) Binding affinity relative to peptide A =1, where affinity greater than 1.0 is "++", 0.51 to 1.0 is "+", 0.1 to 0.5 is "±", and less than 0.1 is "-". K_D of peptide A is approx. 5.6 nM vs DR1 and 42 nM vs DR4w4.

3) Binding affinity relative to peptide B = 1; notation as above. K_D of peptide B is approx.67 nM. Peptide B is the peptide having the sequence set out in Seq. Id. No.2.

4) Inhibition of antigen presentation is relative to peptide of Example 29 = 1, where inhibition greater than 1.0 is "++", 0.51 to 1.0 is "+", 0.1 to 0.5 is "±", and less than 0.1 is "-". The 50% inhibitory dose for the peptide of Example 29 is approx. 10 µM vs DR1, 30 µM vs DR4w4, and 30 µM vs DR4w14.

EXAMPLES 49-50

These examples show that the carboxy-terminal residues can be replaced by dipeptide isosteres. In the peptides below, two C-terminal alanine residues have been replaced by either piperidine-4-carboxyl-amide ($PipNH_2$) or cis-4-aminocyclohexanecarboxylamide ($AccNH_2$) These substitutions do not dramatically alter the DR binding affinities of these peptides relative to the parent peptide. Trans-4-aminocyclo-hexanecarboxylic acid would be equally well tolerated.

Ex.49   ala-Ala-Cha-Ala-Ala-Ala-Ala-Thr-Lys-Ala-Ala-$PipNH_2$
Ex.50   ala-Ala-Cha-Ala-Ala-Ala-Ala-Thr-Lys-Ala-Ala-$AccNH_2$

The results are presented in Table 2, below. The binding assays are performed as described above. The binding affinity of the test peptides is presented relative to either peptide A or peptide B.

TABLE 2

Ex. No.	MHC Binding Assay		
	DR1[1]	DR4w4[1]	DR4w14[2]
49	++	++	++
50	++	++	+

1) Relative to peptide A   2) relative to peptide B

EXAMPLES 51-52

Ex.51   tyr-Ala-Lys-Phe-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Ala-Tol
Ex.52   tyr-Ala-Phe-Gln-Arg-Gln-Thr-Thr-Leu-Lys-Ala-Ala-Tol

These examples (as well as Examples 41,42 and 44-47) show that incorporation of reduced amino acid residues (amino alcohols) at the C-terminus of DR binding peptides does not adversely affect their binding affinities (Table 3).

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The binding assays are performed as described above. The binding affinity of the test peptides is presented relative to either peptide A or peptide B.

TABLE 3

Ex. No.	MHC Binding Assay		
	DR1[1]	DR4w4[1]	DR4w14[2]
51	+	++	++
52	+	++	++

1) Relative to peptide A   2) relative to peptide B

EXAMPLES 53-60

These examples show that R_2 may be replaced by a hydrophobic "end cap" comprising an amino acid mimetic without affecting the binding (Table 4).

Ex.53   Chp-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Phe-ala
Ex.54   Ada-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Phe-ala
Ex.55   Cha-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Phe-ala
Ex.56   Aac-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Phe-ala
Ex.57   Ppa-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Phe-ala
Ex.58   ada-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Phe-ala
Ex.59   2Na-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Phe-ala
Ex.60   1Na-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Phe-ala

Chp = 3-cyclohexylpropionic acid
Ada = L-adamantyl alanine
Cha = L-cyclohexylalanine
Aac = adamantylacetic acid
Ppa = 3-phenylpropionic acid
ada = D-adamantylalanine
2Na = 2-naphthoic acid
1Na = 1-naphthoic acid

TABLE 4

Ex. No.	MHC Binding Assay		
	DR1[1]	DR4v4[1]	DR4v14[2]
53	++	++	++
54	++	++	++
55	++	++	++
56	+	++	++
57	+	++	++
58	+	++	+
59	±	++	++
60	-	++	++

1) Relative to peptide A   2) relative to peptide B

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SEQUENCE LISTING

1) GENERAL INFORMATION:

2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 13 amino acids
        (B) TYPE: amino acid
        (C) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (iii) HYPOTHETICAL: no

    (v) FRAGMENT TYPE: internal fragment

    (vi) ORIGINAL SOURCE:

    (x) PUBLICATION INFORMATION:

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Pro Lys Tyr Val Lys Gln Asn Thr Leu Lys Leu Ala Thr
307   310   315

3) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (iii) HYPOTHETICAL: no

33

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE:

(x) PUBLICATION INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

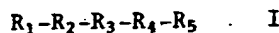Tyr Ala Arg Phe Gln Ser Gln Thr Thr Leu Lys Gln Lys Thr
1   5   10

CLAIMS

1   A peptide of formula I

$$R_1-R_2-R_3-R_4-R_5 \quad I$$

in which, reading from left to right in the direction from the N
to the C terminus;

   R_1 is a spacer sequence of from 0 to 7 amino acid or amino
   acid mimetic residues,

   R_2 is a hydrophobic L-amino or L-amino acid mimetic
   residue,

   R_3 is a spacer sequence of from 2 to 6 L-amino acid or
   L-amino acid mimetic residues,

   R_4 is Thr or a Thr mimetic residue, and

   R_5 is a spacer sequence of between 0 and 10 amino acid or
   amino acid mimetic residues,

provided that at least one residue is a D-amino acid or an amino
acid mimetic.

2.   A peptide according to Claim 1 in which:

   1) R_1 is other than 0 residues and the last residue of R_1,
together with R_2, constitutes an isostere of X_1-Phe; or

   2) R_3 comprises 2-5 residues, two of which constitute an
isostere of X_2-Gln;
wherein X_1 is a L- or D-amino acid mimetic and X_2 can be any
L-amino acid or L-amino acid mimetic.

3.   A peptide according to Claim 1 or Claim 2 in which the last
residue of R_1 (X_1) is Ala or ala.

4.   A peptide acording to Claim 2 in which the isostere of X_2-Gln is
Arg-Gln.

5   A peptide acording to Claim 1 in which the group R_1 contains 1 or
2 residues, composed of ala, ala-Ala, Ac-ala, or Ac-ala-Ala.

6.   A peptide acording to Claim 1 in which the R_2 residue is selected from the group of Tyr, Phe, Trp, Met, and their amino acid mimetics.

7.   A peptide according to Claim 6 in which R_2 is Phe, Tyr, cyclohexylalanine, or adamantylalanine.

8.   A peptide acording to Claim 1 in which R_3 comprises 4 residues, composed of Ala-Ala-Ala-Ala, Ala-Ala-Ala-Arg, or Ala-Ala-Ala-Lys.

9.   A peptide acording to Claim 1 in which R_4 is Thr.

10. A peptide acording to Claim 1 in which R_5 is 3-5 residues long, the last residue being a D-amino acid.

11. A peptide acording to Claim 10 in which R_5 is Ala-Ala-Ala-Ala-ala, Lys-Ala-Ala-Ala-ala, Leu-Lys-Leu-Ala-thr, Leu-Lys-Ala-Ala-ala, or Ala-Lys-Ala-Ala-ala.

12. A peptide acording to Claim 1 which has a core binding region corresponding to R_2, R_3 and R_4 having the sequence Cha-Ala-Ala-Ala-Ala-Thr, Phe-Ala-Ala-Ala-Ala-Thr or Phe-Ala-Ala-Ala-Lys-Thr, where Cha is cyclohexylalanine.

13. A peptide according to Claim 1 having the sequence
     ala-Ala-Cha-Ala-Ala-Ala-Ala-Thr-Lys-Ala-Ala-PipNH$_2$
     ala-Ala-Cha-Ala-Ala-Ala-Ala-Thr-Lys-Ala-Ala-AccNH$_2$
     ala-Cha-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Ala-alaNH$_2$
     ala-Ala-Cha-Ala-Ala-Ala-Lys-Thr-Ala-Ala-Ala-Ala-alaNH$_2$
     ala-Ala-Cha-Ala-Ala-Ala-Ala-Thr-Leu-Lys-Ala-Ala-alaNH$_2$
     tyr-Ala-Ala-Cha-Ala-Ala-Ala-Ala-Thr-Leu-Lys-Ala-Ala-alaNH$_2$
     ala-Ala-Phe-Ala-Ala-Ala-Ala-Thr-Ala-Lys-Ala-Ala-alaNH$_2$, or
     tyr-Ala-Ala-Phe-Gln-Arg-Gln-Thr-Thr-Leu-Lys-Ala-Ala-alaNH$_2$,

     where Cha is cyclohexylamine, PipNH$_2$ is piperidine-4-carboxyl amide, and AccNH$_2$ is cis-4-amino-cyclohexanecarboxamide.

14   A pharmaceutical composition comprising a peptide according to

any one of the preceding claims in association with a pharmacologically acceptable diluent or carrier.

15 A method of treating autoimmune disease in a patient comprising administering an effective dose of a compound according to any one of Claims 1-13.

16 A method according to Claim 15 in which the autoimmune disease is rheumatoid arthritis.

17 A peptide according to any one of Claims 1-13 for use as a pharmaceutical.

18 The use of a peptide according to any one of Claims 1-13 for the preparation of a medicament for the treatment of autoimmune disease.

19 The use of Claim 18 where the autoimmune disease is rheumatoid arthritis.

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5   C 07 K   7/06   C 07 K   7/08   A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
Int.Cl.5	C 07 K A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category°	Citation of Document,¹¹ with indication, where appropriate, of the relevant passages¹²	Relevant to Claim No.¹³
X	WO,A,8606381 (V. BRANTL) 6 November 1986, see page 4, lines 21-28; page 11, lines 17,18, claims ---	1,6,7,9
A	WO,A,9008161 (THE BLOOD CENTER OF SOUTH-EASTERN WISCONSIN) 26 July 1990, see pages 2-4, summary; page 12, example 2; claims ---	1-14,17-19
A	EMBO Journal, vol. 9, no. 6, 1990, Oxford University Press, Oxford, GB; T.S. Jardetzky et al.: "Peptide binding to HLA-DR1: a peptide with most residues substituted to alanine retains MHC binding", pages 1797-1803, see page 1797, abstract; pages 1800-1802, "design and binding..." --- -/-	1-14,17-19

° Special categories of cited documents :¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29-10-1991	18. 12. 91

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	MORTENSEN

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category°	Citation of Document. with indication, where appropriate. of the relevant passages ┊	Relevant to Claim No.
A	Phil. Trans. R. Soc. London, vol. B 323, 1989, London, GB; H.M. Grey et al.: "Structural requirements and biological significance of interactions between peptides and the major histocompatibility complex", pages 545-552, see the whole article -----	1-14,17 -19

FURTHER INFORMATION	NTINUED FROM THE SECOND SHEET	

V. [X] OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons.

1. [X] Claim numbers   because they relate to subject matter not required to be searched by this Authority, namely:

> Remark: Although claims 15-16 are directed to a method
>   of treatment of the human body, the search has
>   been carried out and based on the alleged effects
>   of the compounds.

2. ☐ Claim numbers   because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. ☐ Claim numbers   because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2)) - P9412B 05/91

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 15/11/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8606381	06-11-86	DE-A- 3514587 EP-A,B 0199331 EP-A- 0218650 JP-T- 62501422	30-10-86 29-10-86 22-04-87 11-06-87
WO-A- 9008161	26-07-90	AU-A- 4948890	13-08-90

EPC FORM P0499

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82